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EFFECT OF OZONATION ON ANAEROBIC DIGESTION SLUDGE ACTIVITY AND VIABILITY

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Jaime Chacana[1*], Sanaz Alizadeh[1], Marc-André Labelle[1,2], Antoine Laporte[3], Jalal Hawari[1], Benoit Barbeau[1], Yves Comeau[1]

[1]Polytechnique Montreal, [2]WSP, [3]City of Repentigny

*jaime.chacana@polymtl.ca

ABSTRACT

The effect of ozonation of anaerobic sludge on methane production was studied as a means to increase the capacity of municipal anaerobic digesters. Ozone doses ranging between 0 to 192 mg O_3/g sludge COD were evaluated in batch tests with a bench scale ozonation unit. Ozonation initially, and temporarily, reduced biomass viability and acetoclastic methanogenic activity, resulting in an initial lag phase ranging from 0.8 to 10 days. Following this lag phase, ozonation enhanced methane production with an optimal methane yield attained at 86 mg O_3/g COD. Under these conditions, the yield of methane and the rate of its formation were 52% and 95% higher, respectively, than those measured without ozonation. A required optimal ozone dose could be feasible to improve the anaerobic digestion performance by increasing the methane production potential with a minimum impact on microbial activity; thus, it would enable an increase in the capacity of anaerobic digesters.

*Keywords: A*naerobic digestion, sludge, ozone, extracellular polymeric substances, mechanisms.

1. Introduction

Anaerobic digestion (AD) of primary and secondary sludge is commonly used for sludge reduction, stabilization and energy recovery at municipal water resource recovery facilities (WRRFs) (Appels et al., 2008). Sludge consists of a polymeric network of organic and inorganic compounds, however, its actual composition depends on the source of the sludge (Sheng et al., 2010). The presence of these chemicals, including extracellular polymeric substances (EPS), e.g. polysaccharides, proteins, lipids strongly influence the hydrolysis of sludge during anaerobic digestion (Sheng et al., 2010). The hydrolysis of sludge requires long hydraulic retention times (20 to 30 days), leading to moderate degradation efficiencies (30 to 50%), translating into large volume digesters and high capital expenditures (Foladori et al., 2010a).

Usually, the main factor limiting anaerobic digestion is the hydrolysis of particulate matter. Improving anaerobic digestion through enhancing rate-limiting hydrolysis can increase the degradability leading to improve anaerobic digestion performance (Appels et al., 2008). A variety of treatment techniques have been studied to enhance sludge hydrolysis by using thermal, chemical, mechanical and other biological processes (Appels et al., 2008). Ozonation is one of the preferred chemical treatments, which permits sludge reduction and it is effective in enhancing methane production via the oxidation and solubilisation of sludge (Weemaes et al., 2000). Ozonation of activated sludge prior to anaerobic digestion

(pre-ozonation) effectively enhances its anaerobic biodegradability, but it is not effective with primary sludge (Carrère et al., 2010). Alternatively, the ozonation of digested sludge in the recirculation loop of the anaerobic digester (post-ozonation) has been shown to produce a significant increase in methane production (Battimelli et al., 2003).

Previous studies demonstrated that ozonation has great potential to increase biodegradation of activated sludge (Appels et al., 2008), but other studies showed evidence of biomass destruction (Labelle et al., 2011; Chiellini et al., 2014). Further investigation is required to establish the potential linkage between ozonation of anaerobic digested sludge, methane production, and its biological response. A better understanding of the mechanisms of sludge ozonation and its impact on methane production and biological response may allow for better operational control and design of an anaerobic digestion process integrated with post-ozonation.

The objective of this study was to evaluate the effect of ozonation on the methane production of anaerobic digested sludge, including the mechanisms involved in this process. The specific objectives were to evaluate the impact of ozonation on the methane yield and methane production rate in batch tests, and to evaluate the microbial response of ozonated sludge by monitoring the microbial cell integrity, the metabolism behaviour (key enzyme), the acetoclastic methane activity and the intracellular reactive oxygen species (ROS) formed for various ozone dosages.

2. Material and method

2.1. Sludge ozonation

Anaerobic digested sludge was obtained from the Repentigny WRRF (Quebec) which treats 25 000 m^3/d using a chemically enhanced primary treatment (CEPT) process and stabilizes the sludge in a completely mixed mesophilic (35°C) anaerobic digester with a hydraulic retention time of 19 days. The collected sludge was passed through a 5 mm sieve to remove large debris, and then stored at 4°C until further use.

Ozone was generated by a pure oxygen ozone generator (Peak 2X, Pinnacle, USA). Ozonation of digested sludge was performed in a batch reactor. The gas flow rate was 6 L STP/min with an ozone mass concentration of about 12% by weight. The transferred ozone dose (mg/L) was calculated from the difference between the mass of ozone transferred (mass fed to the reactor minus the mass in the off gas) divided by the volume of sludge. Ozone dosages were normalized as mg O_3/mg COD by dividing the transferred ozone dosage by the initial total COD content of the sample.

Sludge ozonation was conducted on volumes of 2.2 L of digested sludge fed in a 3.8 L column and operated at room temperature. Using a peristaltic pump operating at a flowrate of 6 L/min, the sludge was recirculated through a venturi (484X, Mazzei, USA) where ozone was injected continuously. Higher ozone dosages required longer recirculation time. The contact time ranged from 0.0 to 6.1 minutes for ozone doses between 0 to 192 mg O_3/g COD. Sludge samples were

periodically collected during the operation of the ozonation system. Additionally, a control was prepared to evaluate the effect of treatment without ozone injection.

2.2. Analytical methods

2.2.1. Ozone measurements

The inlet ozone concentration was measured using an ultraviolet ozone meter (BMT 964, BMT Messtechnik GmbH, Germany) while ozone in the off gas was measured using the standard KI method (Rakness, 2005). Dissolved ozone was not measured; it was considered negligible as it was never detected during preliminary tests.

2.2.2. EPS extraction and quantification

EPS were extracted from the control and ozonated samples based on the method of EPS extraction of Liu and Fang. (2002) and Yu et al. (2008). First, 15 mL of the sample was centrifuged at 2 000 g for 15 min at 4°C. The supernatant was collected and filtered (S-Pak 0.45 µm filter, Millipore, USA) to measure soluble EPS. The sludge pellet was re-suspended to its original volume using a phosphate buffer saline (PBS) solution supplemented with 90 µL of formaldehyde (36.5% v/v) and then incubated at 4°C for 1 hour under agitation. The suspension was centrifuged at 5 000 g for 15 min at 4°C and the supernatant was collected and filtered (0.45 µm) for measuring the loosely bound EPS (LB-EPS). The remaining sludge pellet was re-suspended again with a PBS solution to its original volume and incubated for 3 h at 4°C after the addition of 6 mL of a 1 M NaOH solution. The suspension was then centrifuged at 12 000 g for 15 min at 4°C, the decanted

supernatant contained the tightly bound EPS fraction (TB-EPS). The residual sludge pellet was re-suspended once again with a PBS solution to its original volume (pellet fraction).

Proteins and polysaccharides were then measured in the samples before extraction and in soluble EPS, LB-EPS, TB-EPS and pellet fraction. The protein content in the samples was determined using the bicinchoninic acid (BAC) method (Pierce© BCA Protein Assay Kit, Thermo Scientific, USA) with bovine serum albumin (BSA) as the standard. The polysaccharide content of the extracts was analyzed by the phenol-sulfuric acid method using glucose as a standard. Proteins and polysaccharides were measured by a microplate reader (Synergy-HT, BioTek, USA). Excitation–emission matrix (EEM) fluorescence spectra were obtained from the extracts using a luminescence spectrometry (RF-5301pc, Shimadzu, Japan). Samples for EEM analysis were diluted to a final COD of 30 mg COD/L with Milli-Q water. The EEM spectra were collected with the scanning emission spectra (Em) from 220 to 550 nm at 1 nm intervals by varying the excitation wavelengths (Ex) from 220 to 400 nm at 10 nm sampling intervals. Excitation and emission slits were set to 5 nm.

2.2.3. Biochemical methane potential

Methane yield and acetoclastic activity were evaluated by measuring the biochemical methane potential (BMP) in 160 mL serological bottles incubated at 35°C based on Saha et al. (2011). A gas manometer (DG25, Ashcroft, USA) was used to measure the biogas production and the methane gas content was quantified with a gas chromatograph (GC-456, Bruker, USA) equipped with a

thermal conductivity detector (150°C). The modified Gompertz model was applied to the cumulative methane production data to determine the maximum methane production rate in the samples (Lay et al., 1996). Methane yield was evaluated without substrate addition, and the acetoclastic activity test was fed with sodium acetate solution. The methane production was evaluated at the standard temperature and pressure (STP) of 0°C and 1 atm.

2.2.4. Characterization of biological response

Bacterial viability of anaerobic sludge was evaluated using the Live/Dead *Baclight* bacterial viability kit (Molecular Probes, Invitrogen, Kit L13152) and the microplate reader (Synergy-HT, BioTek, USA) using the modified protocol of Chen et al. (2012). The fluorescence intensity of the stained bacterial suspensions (F_{cell}) was determined at an excitation of 488 nm and detection at 635 nm (red) and 530 nm (green), for red-fluorescent nucleic acid stain propidium iodide (PI) and green-fluorescent nucleic acid stain SYTO 9, respectively. The green/red fluorescence ratios ($R_{G/R}$) were used to compare the bacterial inactivation triggered by different doses of ozone. Different proportions of fresh sludge (optimal viable cells) and positive control, inactivated cells with alcohol treatment (2-propanol, 70%), were used as standards. The viability calibration curve was obtained by linear regression of the green/red fluorescence ratio ($R_{G/R}$) vs the percentage of viable cells.

The dehydrogenase activity was quantified by the protocol described by Von Mersi and Schinner (1991). The technique uses soluble and colorless 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) reduction to the red insoluble iodonitrotetrazolium formazan (INF) as a tracer of active bacterial

157 electron transport systems (Caravelli et al., 2004). Briefly, triplicates of sludge
158 samples (0.5 g) were spiked with 0.75 mL of TRIS buffer (1M; pH 7.0) and 1 mL of
159 0.5% INT solution (9.88 mM), slightly mixed using a vortex for 30 seconds. After 2
160 hours incubation at 40 °C in the dark, the intracellular INF crystals were extracted
161 with 5 mL ethanol/N,N-dimethylformamide solution (1/1 v/v) and incubated for 1 h
162 at 40 °C in the dark. The concentration of developed formazan in the retained
163 supernatant of sludge was determined by a UV/vis spectrophotometer at 464 nm
164 using the extraction solution, ethanol/N,N-dimethylformamide solution (1/1 v/v) as
165 reference blank. INT-electron transport system activity was calculated using the
166 modified equation proposed by Yin et al. (2005) (equation 1)

$$\text{INT–ETSA} = D_{464} \cdot V / k_i \cdot W \cdot t \qquad (1)$$

167 Where INT-ETSA is the INT-electron transport system activity (mg INTF/g
168 biomass/h), D_{464} is the absorbance of the supernatant at 464 nm; V is volume of
169 solvent (mL), k_i is the slope of standard curve of absorbance at 485 nm vs INTF
170 concentration (O.D. mL/mg INTF), W is the weight of biomass (g) and t is the
171 incubation time (h).

172 ROS was determined using an established fluorescence assay (You et al., 2015).
173 The sludge samples were rinsed three times with 0.1 M phosphate buffer (pH 7.4)
174 and the pellets were re-suspended in 0.1 M phosphate buffer containing 50 μM
175 dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes, Invitrogen).
176 The resulting mixture was incubated at 25 ± 1°C in the dark for 30 min. The

177 generated fluorescent fluorescein DCF was measured using a microplate reader
178 (Synergy-HT, BioTek, USA) at excitation of 488 nm and emission of 525 nm.

179 2.2.5. Other analytical methods

180 Chemical oxygen demand (COD) was measured using the HACH method (HACH
181 Reactor Digestion Method 8000). Soluble COD was determined on centrifuged
182 (10 000 g, 10 min) and filtered (S-Pak 0.45 µm filter, Millipore, USA) samples.

183 The morphology of blank and ozonated sludge were visualized using a scanning
184 electron microscope (SEM, JEOL JSM7600F). The sample preparation procedure
185 was adapted from Sheng et al. (2011). Sludge sample preparation included the
186 fixation with 2.5% glutaraldehyde in phosphate buffer for 30 min, followed by serial
187 ethanol dehydration. The gold-coated samples were observed with a high-
188 resolution SEM equipped with a field emission gun at a resolution of 1.4 nm at 1 kV
189 and an accelerating voltage of 0.1 to 30 kV.

190 2.6. Statistical analysis

191 Anaerobic biodegradability tests and EPS extraction were conducted in duplicate,
192 3D-EEM tests without replication and the other analyses in triplicate. The Student's
193 t-test was used to compare the quantitative variables considering a p value < 0.05
194 to be statistically significant. A nonlinear optimization by least squares procedure
195 was applied to calculate the maximum methane production by the Modified
196 Gompertz model (Lay et al., 1996).

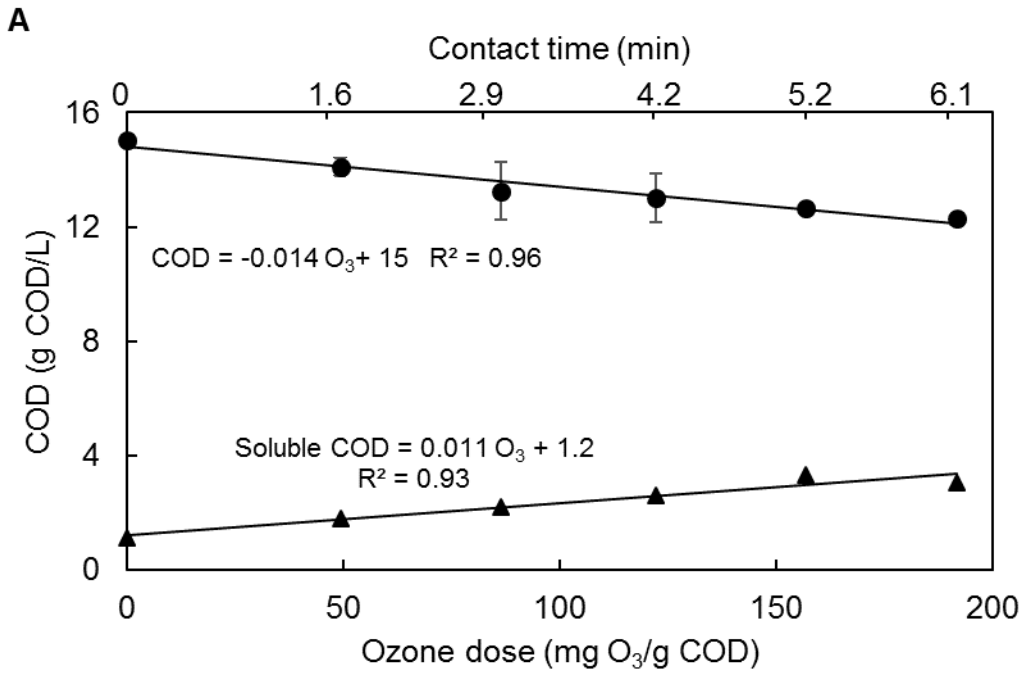
197

198 **3. Results and discussion**

199 3.1. Effect of ozonation on COD solubilization and mineralization

200 The impact of ozonation on total COD was shown in Figure 1A. During ozonation,
201 total COD was reduced from 15.0 to 12.3 g COD/L. This was a decrease of
202 approximately 18% at 192 mg O_3/g COD. The decrease of COD by ozonation
203 could be attributed mostly to the complete oxidation of a portion of the organic
204 compounds to CO_2 and water (mineralization); this is based on previous studies for
205 ozonation of activated sludge that reported a decrease of total organic carbon
206 (TOC) similar to the reduction of COD, and also an increase of CO_2 in the residual
207 gas of ozone reactor (Weemaes et al., 2000; Déléris, 2001).

208 Soluble COD increased significantly from 1.13 to 3.31 g COD/L (157 mg O_3/g
209 COD) during ozonation, representing a solubilization of 15.7% (Figure 1A). Higher
210 ozone doses resulted in an apparent decrease in the solubilized COD which may
211 be due to increased mineralization. Solubilization effects observed in this study are
212 consistent with the study of Weemaes et al. (2000) who reported 29% increase in
213 COD solubilization of sludge exposed to 200 mg O_3/g COD. A comparison of the
214 efficiency of sludge solubilization and mineralization in different studies is difficult
215 since the performance depends on several factors including ozone injection
216 conditions, ozone dosage and sludge characteristics (Foladori et al., 2010a). No
217 significant solubilization and COD decrease were observed in the control.



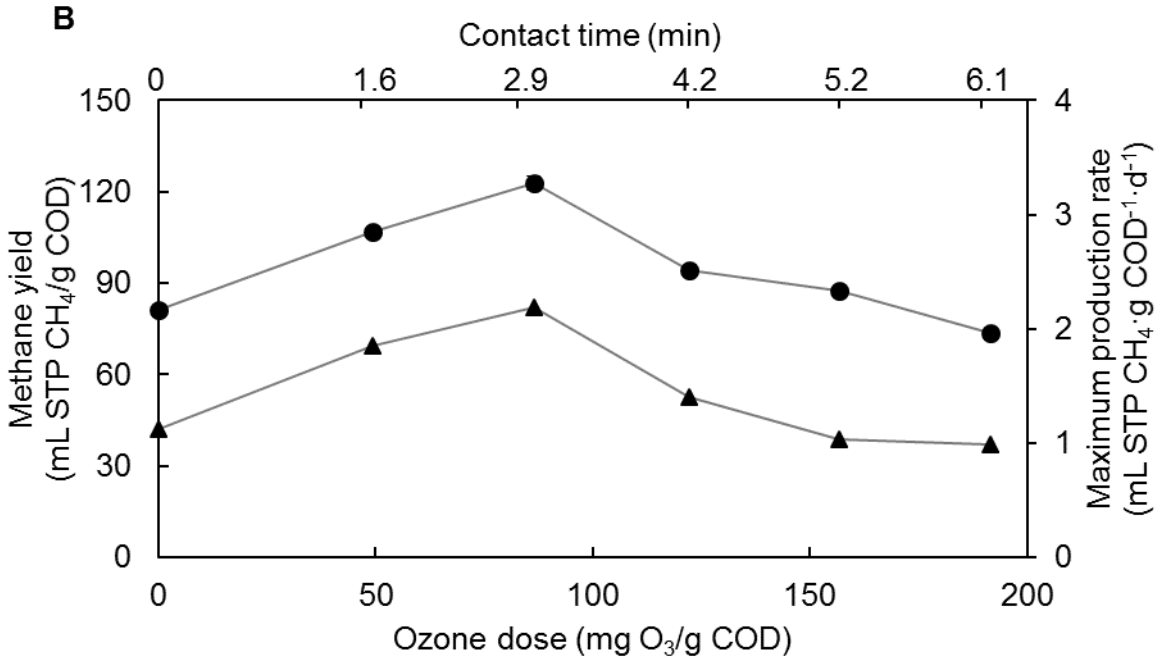


Figure 1: Effect of ozone dose and contact time on COD and methane production – (A) total COD (●) and (▲) soluble COD; (B) Methane yield of ozonated sludge (●) and Gompertz maximum production rate (▲).

3.2. Effect of ozonation on methane production

The efficiency of ozonation on methane yield was evaluated in BMP assays using ozonated sludges and controls (Figure 1B). Ozonation leads to a significant increase in methane production and reaching a maximum yield of 123 mL STP CH_4/g COD for an ozone dose of 86 mg O_3/g COD. In the absence of ozone, methane production did not exceed 81 mL STP CH_4/g COD. The composition of the biogas was not impacted significantly during ozonation. The average composition of the biogas in both ozonated sludges and controls was 71.3%, 28.6, and 0.05% for CH_4, CO_2 and H_2, respectively. These experimental findings demonstrated that ozonation could increase methane production. Interestingly, using doses of ozone higher than 86 mg O_3/g COD reduced the improvement in methane production. Similar behavior was reported by Weemaes et al. (2000), who found an optimal methane production for an ozone dose of 100 mg O_3/g COD (80%), but also a higher ozone dose reduced the possitve effect on methane production (30%) for activated sludge mixed with primary sludge.

The maximum methane production rate of samples was determined by fitting the cumulative methane production data to the modified Gompertz model (Lay et al., 1996). A good agreement between the experimental data and the modified Gompertz model (R^2>0.95) was obtained. The maximum methane production rate was 2.2 mL STP $CH_4 \cdot g\ COD^{-1} \cdot d^{-1}$ for an ozone dose of 86 mg O_3/g COD, representing an increase of 94.5% relative to the untreated sludge (Figure 1B). Ozone doses between 122 to 192 mg O_3/g COD did not change significantly the maximum methane production rate compared to the untreated sample. The

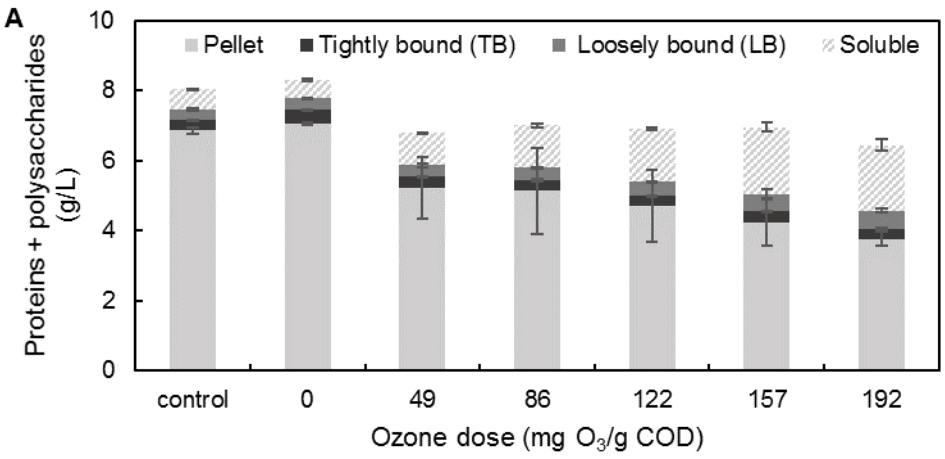
maximum methane production rates of the current study are low compared to Weemaes et al. (2000). These authors observed a methane production rate of 4.3 mL STP $CH_4 \cdot g\ COD^{-1} \cdot d^{-1}$ for untreated sludge, while for the optimal ozone dose, the production rate was 9.1 mL STP $CH_4 \cdot g\ COD^{-1} \cdot d^{-1}$. This difference may be due to the type of sludge used. Digested sludge has a low biodegradability since the anaerobic digester has already removed readily biodegradable matter.

Ozonation can induce the release of soluble substances into the aqueous phase, this phenomenon increases the accessibility of compounds to microorganisms, and therefore, improves the anaerobic biodegradability of ozonated samples. The maximum ozone dose tested (192 mg O_3/g COD) reduced methane yield and the methane production rate, probably due to the complete oxidation of solubilized matter caused by the mineralization. Therefore, mineralization should be minimized, while organic matter solubilization should be maximised to enhance methane production (Weemaes et al. 2000; Carballa et al., 2007).

3.3. Effect of ozonation on EPS

The effect of ozonation on the protein and polysaccharide content from different extracted EPS fractions and pellets of anaerobic digested sludge is shown in Figure 2A. For the un-ozonated sludge, the total content of proteins and polysaccharides were 6.6 and 1.8 g/L, respectively, with almost 85% of both polymer substances found in the pellet remaining after centrifugation, while the bound EPS and soluble EPS accounted for only 8.6% and 6.2%, respectively. The ratio of proteins and polysaccharides of extracted EPS (soluble EPS and bound

EPS) was 1.84, as compared with the reported ratios of 1.1 to 2.8 for digested sludge (Morgan et al., 1990).



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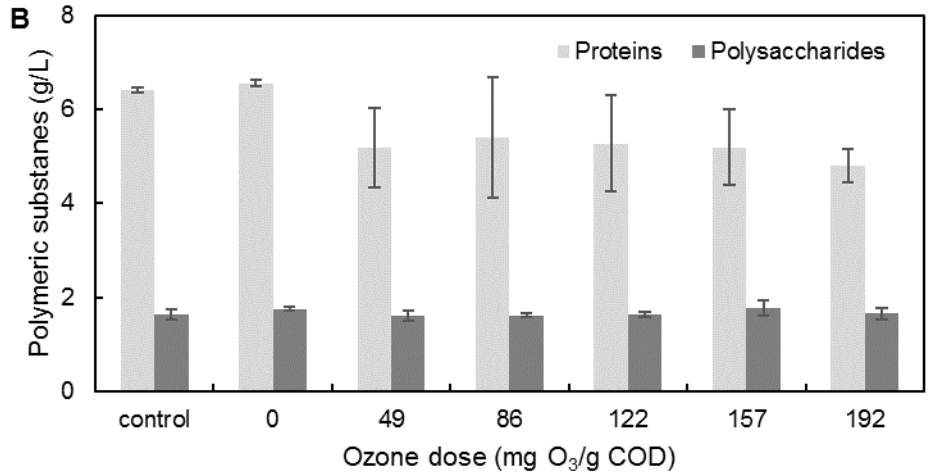

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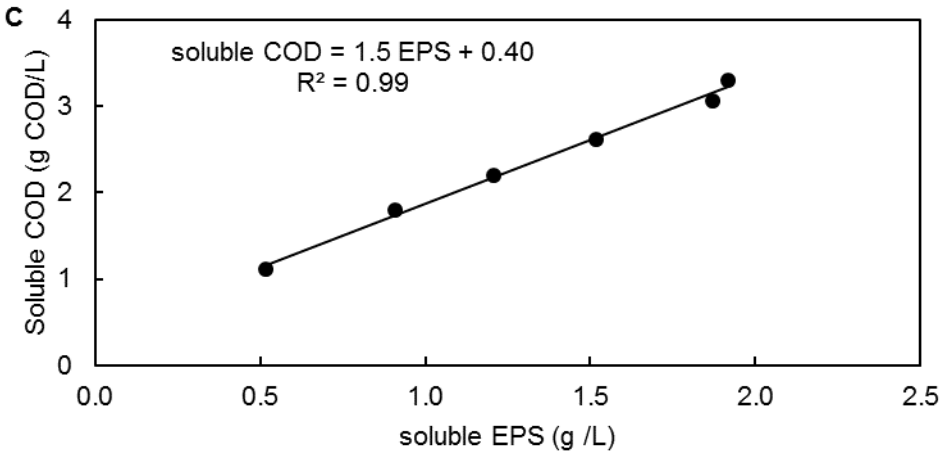

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276 Figure 2: (A) Determination and distribution of EPS (proteins and polysaccharides)
277 in extracted EPS fractions and pellet of digested sludge for an ozone dose
278 between 0 to 192 mg O_3/g COD, (B) effect of ozonation on protein and
279 polysaccharide content, (C) Correlation between soluble EPS and soluble COD.

280 A non-significant change in protein concentration was observed for ozone doses
281 between 0 to 157 mg O_3/g COD (Figure 2B). However, the protein content was
282 reduced by 27% for an ozone dose of 192 mg O_3/g COD. Oxidation can cause
283 structural modification of proteins ranging from fragmentation of the polypeptide
284 backbone to aggregation by cross-linking between amino acid residues (Davies,
285 2005). Furthermore, ozone can oxidize amino acid residues, such as cysteine,
286 tryptophan and tyrosine (Cataldo, 2003; Meng et al., 2016) which should usually be
287 quantified by the BCA method (Wiechelman et al., 1988). However, the by-
288 products of oxidation could not be quantified as proteins.
289 As for polysaccharides, no significant decrease in content was noted for doses up
290 to 192 mg O_3/g COD (Figure 2B). Polysaccharides were reported to react weakly
291 with ozone (Bablon et al., 1991). This is expected knowing that proteins have more
292 reactive functional groups (-NH_2, -SH, -COOH, amide linkages) than
293 polysaccharides (mostly –OH and ether linkages). Ozonation of β-D-glycosidic
294 linkages in polysaccharides leads to selective depolymerisation into short chain
295 polysaccharides and oligosaccharides (Wang et al., 1999). Using the phenol-
296 sulfuric acid method, these oligosaccharides will be detected as polysaccharides,
297 thereby, the total sugar content will remain constant.
298 For the pellet residues, measured amounts of proteins and polysaccharides were
299 significantly reduced during ozonation from 7.1 to 3.8 g/L at 192 mg O_3/g COD.
300 Total content of proteins and polysaccharides reduced from 8.3 to 6.5 g/L using an
301 ozone dose of 192 mg O_3/g COD. TB-EPS, LB-EPS and soluble-EPS content of
302 the sludge changed significantly upon exposure to ozone compared to the non

303 ozonated sample. TB-EPS decreased from 0.37 to 0.29 g/L for an ozone dose of
304 192 mg O_3/g COD whereas the amount of LB-EPS and soluble-EPS increased
305 linearly from 0.34 to 0.52 g/L (R^2 = 0.71) and 0.52 to 1.9 g/L (R^2 = 0.98),
306 respectively.

307 Ozonation was found to have a significant effect on the distribution of proteins and
308 polysaccharides in various fractions of the digested sludge. Initially, 85% of
309 proteins and polysaccharides were concentrated in the pellet fraction, but after
310 ozonation 59% remained in the pellet (192 mg O_3/g COD). On the other hand,
311 proteins and polysaccharides in the soluble fraction increased from 6.2 to 29%
312 after ozonation (192 mg O_3/g COD).

313 During ozonation, the concentration of EPS in the soluble layer increased while the
314 amount of proteins and polysaccharides from the pellet was reduced as the ozone
315 dose was increased suggesting that ozonation causes the release of EPS from the
316 inner layer to the outer layer. Protein release to the soluble phase was higher than
317 that of polysaccharides. The increase in EPS content in the soluble layer correlated
318 with the COD solubilization (Figure 2C). These results suggest that ozonation
319 disintegrates sludge flocs and releases COD, proteins and polysaccharides from
320 the pellet into the soluble phase. The control showed that mechanical friction of the
321 pump did not cause any significant effect on the protein and polysaccharide
322 content and its distribution in the different fractions.

323 Three-dimensional EEM spectroscopy was applied to characterize the EPS
324 extracted from untreated and treated sludge (192 mg O_3/g COD). Peaks at four
325 different locations were identified according to the literature (Chen et al., 2003).

The fluorescence peak positions and fluorescence intensity of the different EPS fractions are detailed in Table 1 and Figure S1 (Supplementary Information). The peaks were associated with the presence of aromatic amino acids, e.g. tryptophane in proteins (peak A), fulvic acid-like (peak B), soluble microbial by-products-like (peak C) and humic acid-like (peak D). EEM intensities of peaks tended to decrease after ozonation. Intensity reduction of the fluorescence peaks can be an indication of oxidation and removal of some of the molecular functionalities responsible for fluorescence. Although protein content increased in soluble EPS and LB-EPS, tryptophan and tyrosine are susceptible to oxidation by ozone, thus, reducing the intensity of fluorescence peaks A and C (Figure S1).

Table 1: Impact of ozonation on peak intensities of the fluorescence spectra for soluble EPS, LB-EPS, and TB-EPS fractions of anaerobic digested sludge (A = tryptophan, B = fulvic acid-like, C = soluble microbial by-products-like, and D=humic acid-like)

EPS fractions	Ozone dose mg O_3/g COD	Peak intensities A	B	C	D
Soluble	0	340	1000	270	880
	192	200	540	140	720
LB-EPS	0	220	440	200	300
	192	67	180	140	180
TB-EPS	0	860	970	910	570
	192	490	590	650	430

3.4. Observations of samples by scanning electron microscopy

SEM observations revealed a distinct difference in the morphology of the control and the ozone treated sludge floc (Figure S2, Supplementary Information). The untreated sludge samples consisted of smooth, dense and integrated structures, with embedded cells in the sludge matrix. As the ozone dose increased, more irregular porous and rough surface structures were observed in the treated samples. Surface deformation and sludge floc disaggregation were observed in sludge samples treated with a dose higher than 86 mg O_3/g COD. The morphology modification of sludge agrees with the alteration of sludge properties, such as EPS, which was confirmed by the release of soluble proteins.

3.5. Effect of ozonation on viability, enzymatic activity, ROS production and acetoclastic activity of anaerobic sludge

3.5.1. Viability and dehydrogenase activity assay

Modified microbial activity of anaerobic sludge following ozonation was characterized by the determination of the biomass viability and the dehydrogenase activity (Figure 3A). The primary ozone dose of 49 mg O_3/g COD inhibited by 57% the relative viability of cells. Ozone treatment between 49 and 122 mg O_3/g COD significantly tailed off the viable biomass with intact membrane, coupled with a higher ratio of inactivated cells. The ozone treatment at doses higher than 157 mg O_3/g COD resulted in significant lysis of biomass with a relative viability of less than 5%. Therefore, significant inactivation of active biomass was observed by ozonation at all tested doses. Membrane integrity defines the potential metabolic activity of the intact cells; therefore, cells with damaged membranes can be classified as permeabilized/dead cells (Foladori et al., 2010b). The influence of

364 ozonation on bacterial viability consists of progressive degradation initiated with the
365 physical alteration of membrane permeability and cell integrity, followed by the lysis
366 reaction (Thanomsub et al., 2002). The bacterial cell membrane is comprised
367 dominantly of lipids with abundant C=C double bonds as well as proteins (Winter et
368 al., 2008; Arts et al., 2015). Ozone is a strong electrophile and thus, can easily
369 react with unsaturated lipids via their nucleophilic –C=C– functionality leading to
370 cellular membrane decomposition and the release of cellular components,
371 including EPS. It has been reported that oxidation of C=C double bonds in lipids
372 forms malondialdehyde (MDA) (Han et al., 2016) causing decomposition of the
373 cellular membranes resulting in cell disruption and subsequent leakage of cellular
374 contents (Foladori et al., 2010b).

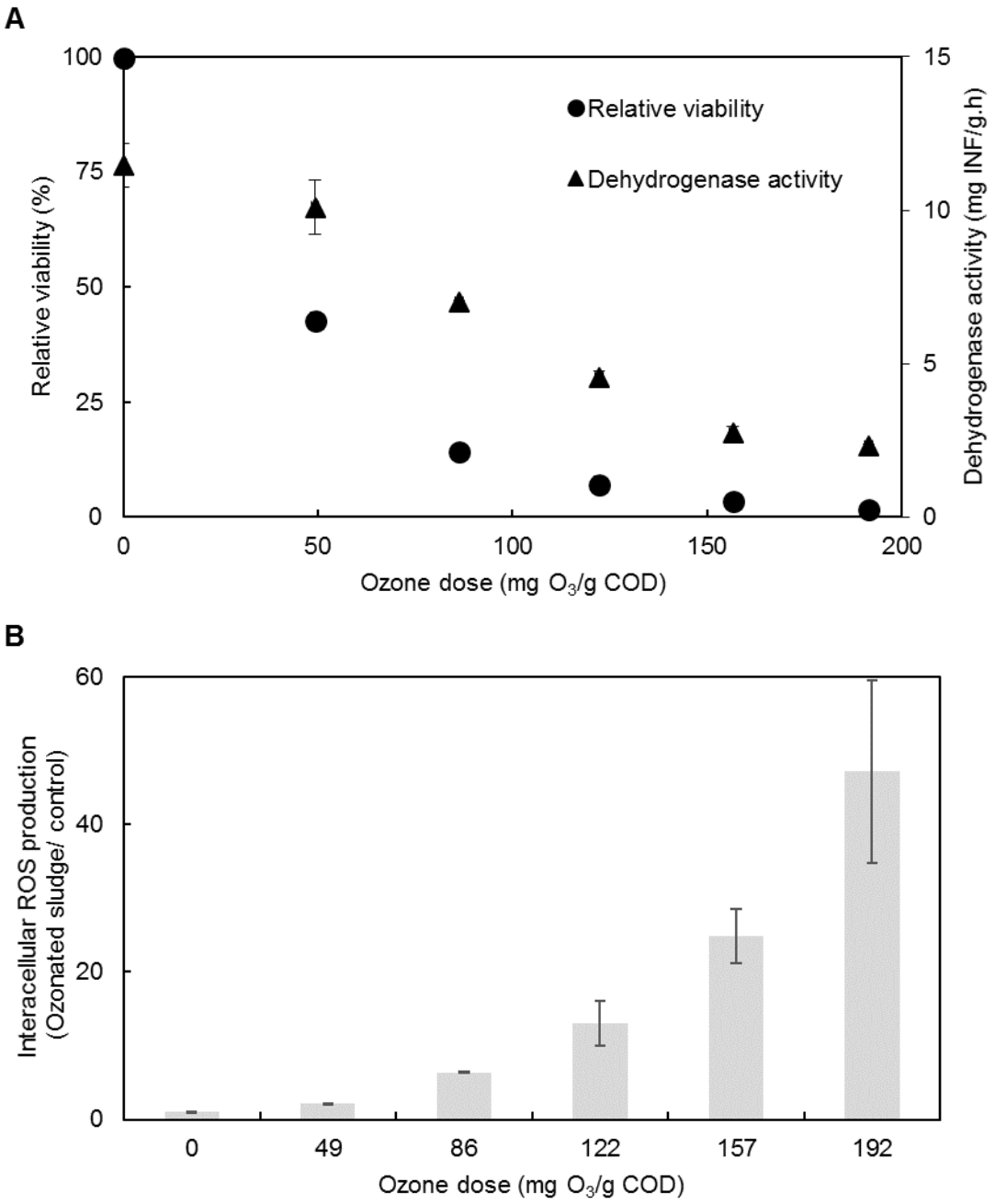


Figure 3: Effect of ozone dose on (A) relative viability, dehydrogenase activity, and (B) intracellular ROS production.

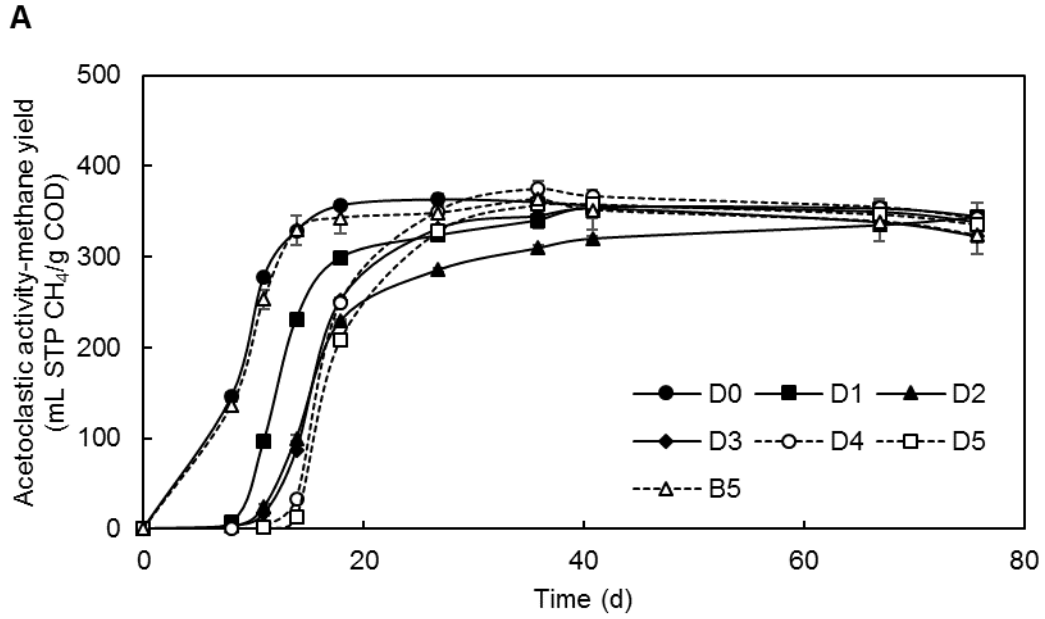
3.5.2. Intracellular ROS production

Ozonation induced ROS in treated sludge at each ozone dose (Figure 3B). Intracellular ROS augmented with an increase in ozone dosage. The ROS concentration was 46 times higher than the control at the highest ozone concentration of 192 mg O_3/g COD. The phenolic and olefinic groups and proteins in the lipid bilayers of the bacterial cell wall are the primary oxidative sites leading to the formation of ROS, such as hydroxyl radicals ($OH^{\cdot}$), peroxides ($RCOO^{\cdot}$) and superoxide radical anions ($O\text{-}O^{\cdot -}$) (Pryor et al., 1991). Subsequent reactions of ROS with cellular components, such as lipids, proteins and nucleic acid leads to cell disruption and decomposition and causing the release of intracellular components (Baier et al., 2005). Thus, the significantly higher intracellular ROS above 86 mg O_3/g COD confirms the potential of oxidative stress to trigger cell membrane damage and enzyme inhibition for ozonated sludge.

3.5.3. Acetoclastic methane activity

The acetoclastic methanogenic activity of sludge was used to determine the effect of ozonation on the anaerobic biodegradability of sludge. The acetoclastic methanogenic yield of control and ozonated sludge are illustrated in Figure 4A. Acetoclastic activity after short-term exposure to ozone showed a lag phase, which increased as ozone dose increased. The initial inhibition of acetoclastic activity was consistent with the significant decrease of dehydrogenase enzymatic activity and loss of intact viable cells measured at the beginning of experiment. Similarly, the complete inhibition of respiratory activity of activated sludge has been reported at 100 mg O_3/g TSS (Chu et al., 2008). However, approximately 95% of the

403 theoretical methane production (350 mL STD CH_4/g COD) was achieved in the
404 samples over 14 days despite the presence of a lag phase of 0.8 to 10 days in the
405 initiation of activity for all ozonated sludge. Furthermore, the dehydrogenase
406 activity of sludge increased during the incubation (192 mg O_3/g COD) (Figure 4B).
407 The cell membrane disintegration, alteration of permeability and interaction of
408 membrane proteins and lipids with ozone can inhibit the acetoclastic activity of
409 sludge. The extension of the activity test, up to 80 days, demonstrated the recovery
410 of microbial activity of ozonated sludge due to the potential recovery of the
411 bacterial community.



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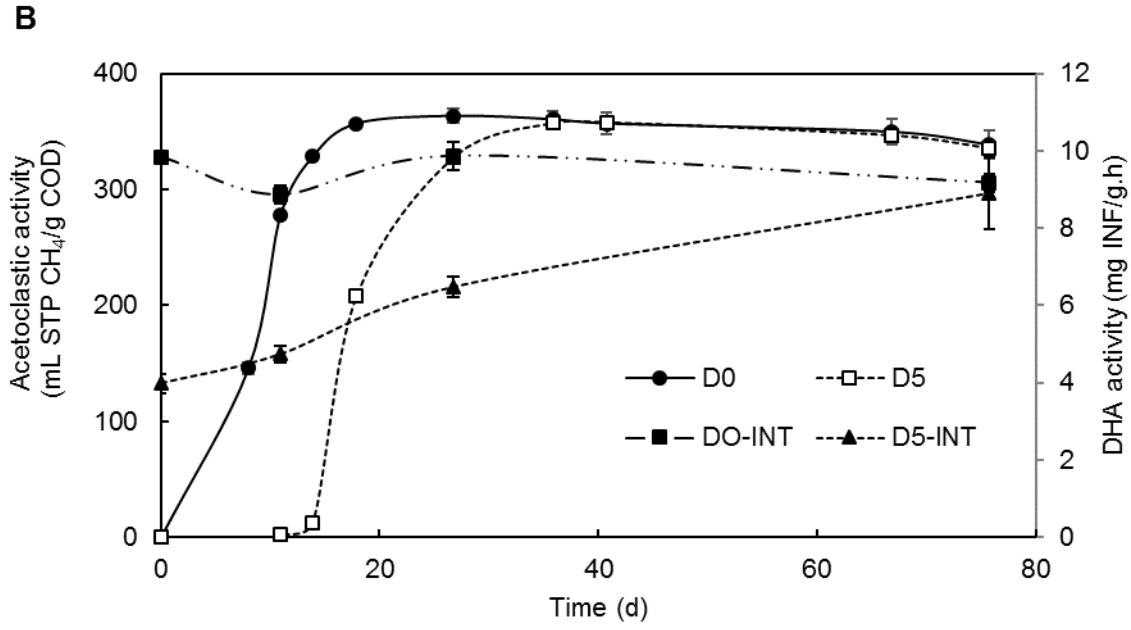

413

414 Figure 4: (A) Impact of exposure to ozone on acetoclastic methanogenic activity of
415 anaerobic sludge, and (B) comparison of dehydrogenase activities and acetoclastic
416 activity for D0 and D5 (D0=0 mg O_3/g COD, D1=49 mg O_3/g COD, D2=86 mg O_3/g
417 COD, D3=122 mg O_3/g COD, D4=157 mg O_3/g COD, D5=192 mg O_3/g COD, B5=
418 control).

419

3.6. Potential mechanisms of improving of anaerobic biodegradability

Ozonation was shown to increase the solubilization of sludge mainly via partial disintegration/solubilization of the sludge matrix and damage to the cell membrane integrity. Ozonation can disintegrate the sludge matrix and release COD, proteins and polysaccharides from the pellet into the soluble phase, thereby, promoting the enhancement of methane production during anaerobic digestion. Furthermore, the reduction in viability of the sample suggests that the broken cells can release intracellular matter into the solution. The enhancement in methane production may not only be ascribed to solubilization and it also can be influenced by the increase of the biodegradability of organic products generated during ozonation, e.g. the products of oxidation by ozone of olefins and aromatic compounds are more biodegradable than their parent compounds (Hübner et al., 2015). As a result of the increase in solubilization and biodegradability, anaerobic degradation can be enhanced, improving methane yield and accelerating digestion time. An overdose of ozone can reduce the methane production potential, probably due to the potential mineralization of the solubilization matter. Additionally, an overdose of ozone can minimize the viability of anaerobic biomass and enzymatic activity which could have a negative impact on the stability of anaerobic digesters in a post-treatment configuration.

4. Conclusions

The effect of ozonation on anaerobic digested sludge and its impact on microbial response were evaluated by monitoring methane production, EPS, microbial activity, viability and ROS. The EPS matrix was impacted by ozonation, resulting in the release of COD, proteins and polysaccharides into the soluble phase. Ozonation, initially and temporarily, reduced biomass viability and activity, but following this lag phase, ozonation enhanced methane production. The optimized ozone dose of 86 mg O_3/g COD increased the methane yield up to 52% and the methane production rate up to 95%. Therefore, ozonation could be used to increase the capacity of anaerobic digesters.

Acknowledgements

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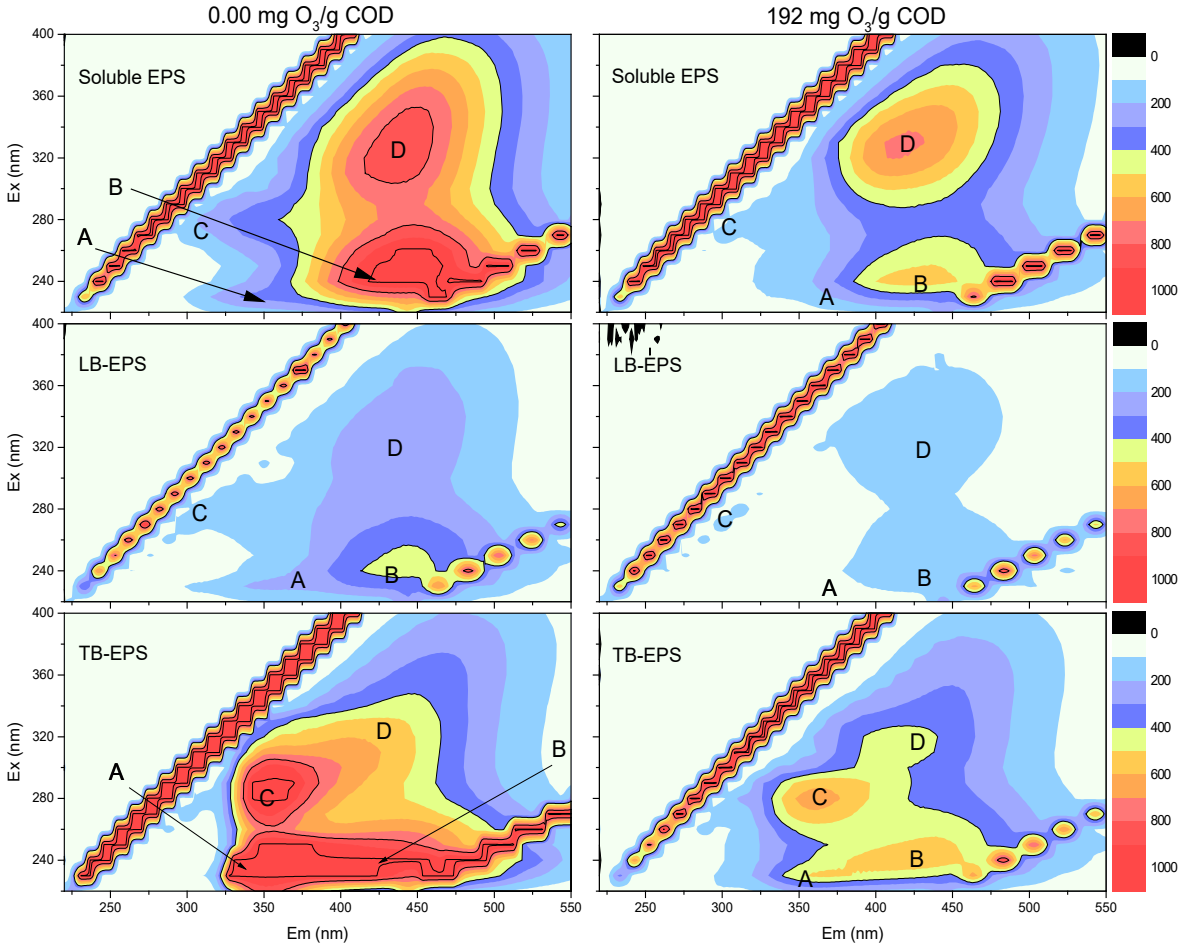
582 **Appendix. Supplementary information**



583
584 Figure S1: EEM spectra of the extracted EPS fractions for untreated and treated

585 sludge (192 mg O_3/g COD).

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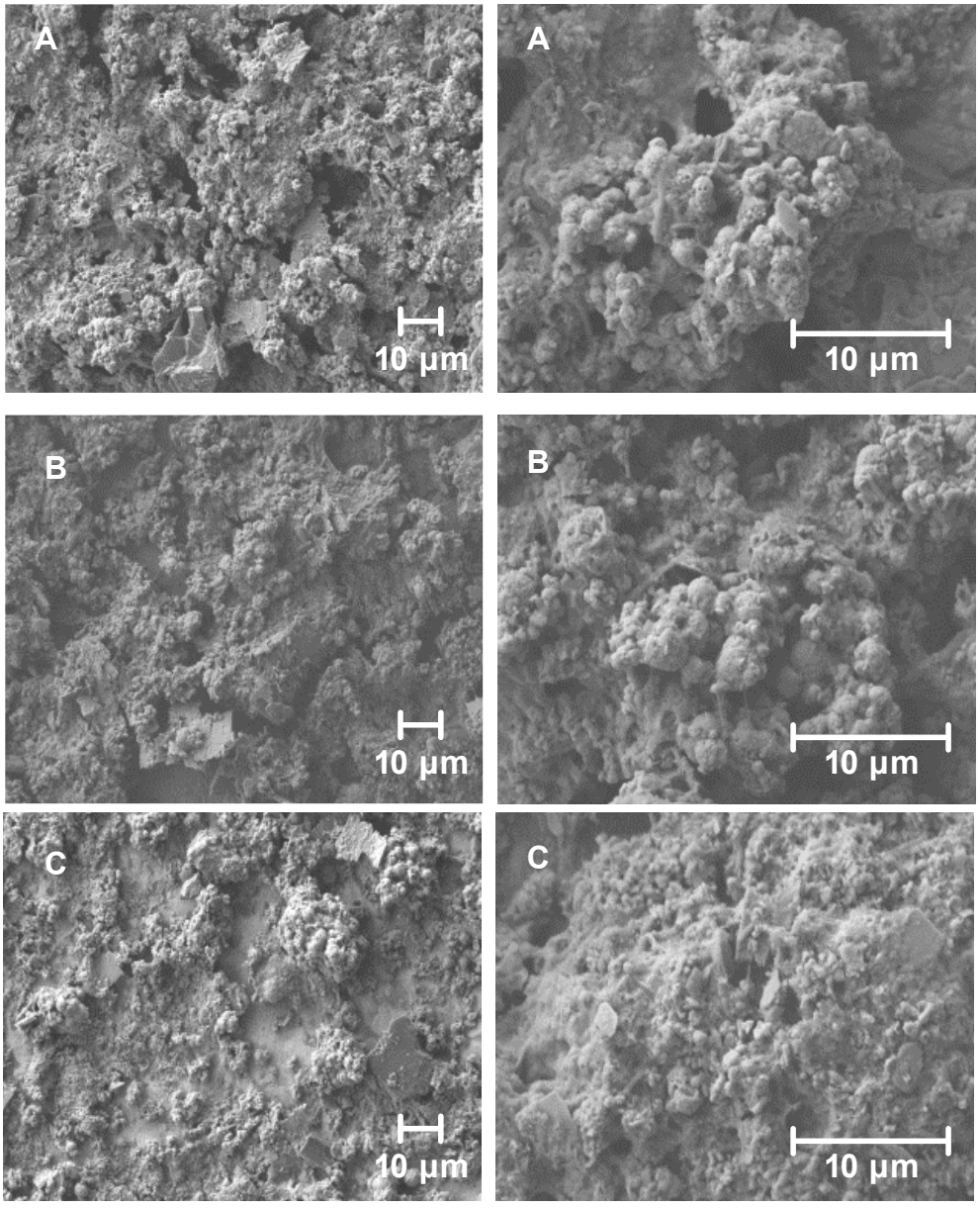

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588 Figure S2: Scanning electron micrographs imaging of anaerobic sludge exposed to 0 mg O_3/g COD (A), 86 mg O_3/g COD (B), and 192 mg O_3/g COD (C).